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Analysis of a *De novo* Complex Chromosome Rearrangement Involving Chromosomes 4, 11, 12 and 13 and Eight Breakpoints by Conventional Cytogenetic, Fluorescence *In situ* Hybridization and Spectral Karyotyping

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A complex chromosome rearrangement (CCR) with eight breakpoints resulting in four derivative chromosomes (4, 11, 12 and 13) was detected prenatally in a male fetus of a twin pregnancy. The karyotype of the female second fetus was normal. The apparently balanced *de novo* CCR was identified by classical cytogenetic methods and fluorescence *in situ* hybridization (FISH). We compared these findings with results from spectral karyotyping (SKY).

KEY WORDS: complex chromosome rearrangement (CCR); prenatal diagnosis; fluorescence *in situ* hybridization (FISH); spectral karyotyping (SKY)

INTRODUCTION

Complex chromosome rearrangements (CCRs) have been defined as involving more than two chromosome breaks with reciprocal exchange of segments between these chromosomes (Pai *et al.*, 1980). When the number of chromosomes—up to seven (Kousseff *et al.*, 1987), and the number of breakpoints—up to 10 (Tupler *et al.*, 1992), increases, it becomes more and more difficult for the cytogeneticist to characterize the rearrangements correctly.

The interpretation of CCRs by conventional banding techniques alone may be impossible, especially when deletions, insertions or inversions are present in addition to the reciprocal translocations. Fluorescence *in situ* hybridization (FISH) with chromosome specific DNA probes allows the study of chromosome translocations in more detail (Batista *et al.*, 1993; Fuster *et al.*, 1997; Wieczorek *et al.*, 1998). New multicolour FISH techniques like spectral karyotyping (SKY) created the possibility to analyse the whole genome in one hybridization. With SKY, a simultaneous colour differentation is achieved by specific combinatorial labelling of 24 human chromosomes with five different fluorochromes and measurement of the entire spectrum through a single custom designed filter and spectroscopic analysis (Schröck *et al.*, 1996).

We report a *de novo* complex rearrangement with eight breakpoints involving four chromosomes which was detected prenatally in a male fetus of a twin pregnancy.

CASE REPORT

In the first pregnancy of a healthy couple, amniocentesis of a twin pregnancy was performed in the 17th week because of advanced maternal age (36 years). The course of pregnancy had been uneventful up to this time. A normal female karyotype (46,XX) and a male karyotype with four derivative chromosomes (46,XY, t(4;11;12;13)) were diagnosed. For confirmation of the diagnosis and to obtain material for additional investigations fetal blood sampling was performed. The additional chromosome analyses confirmed the previous findings.

Ultrasonographic investigations during the 21th gestational week showed roughly normal fetal growth parameters. No morphologic abnormalities were discovered. A second ultrasonographic study one week later revealed a beginning growth retardation, hectic abnormal movements and retrognathia in the male fetus.

On account of the karyotype with eight breakpoints in four involved chromosomes the risk for phenotypic abnormalities was estimated to be between 10 and 20 per cent because of possible microdeletions and microduplications. The suspicion of abnormal development of the male fetus was confirmed by the ultrasonographic peculiarities of the male fetus compared with the female one.

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Selective fetocide of the male fetus was carried out in the 24th gestational week. A healthy girl and a fetus papyracaeus were born at term. The girl is now two years old and has developed according to age.

METHODS

Karyotyping

Metaphase chromosome preparations were obtained from amniotic fluid cell cultures and PHA stimulated fetal lymphocytes from both fetuses using standard methods. Chromosome analyses of the parents were carried out on PHA stimulated lymphocyte cultures from peripheral blood. Chromosome banding was performed using the trypsin-Giemsa technique of Seabright (1971). Nucleolus organizer regions (NOR) were visualized by silver nitrate staining according to Benn and Perle (1992).

Fluorescence *in situ* hybridization (FISH)

Slides were hybridized with whole chromosome paints of chromosomes 4 (Oncor-Appligene, Gaithersburgh, VA, U.S.A.), 11 and 12 (Angewandte Gentechnologie Systeme Heidelberg, Germany) according to the manufacturer's instructions and the protocol of Lichter *et al.* (1988). Chromosomes were analysed on a Leitz Diaplan epifluorescence microscope and documented with 320T Kodak Ektachrome slide film.

Spectral karyotyping (SKY)

Chromosome painting probes generated from flow sorted human chromosomes using sequence independent DNA amplification were labelled in a secondary PCR reaction by directly incorporating haptenized or fluorochrome-conjugated nucleotides as described by Schröck *et al.* (1996). In the presence of an excess of human Cot-1 DNA (BRL) the differentially labelled chromosome painting probes were combined and precipitated.

Slides were hybridized for 2 days at 37°C. The biotinylated probe sequences were visualized using avidin Cy5 (Amersham Life Sciences), and the digoxigenin-labelled probe sequences by incubation with an anti-mouse digoxin antibody (Sigma Chemicals) followed by a goat anti-mouse-antibody conjugated to Cy5.5 (Amersham Life Sciences). Chromosomes were counterstained with DAPI and covered in paraphenylene-diamine solution (Sigma).

Image acquisition was performed using a SD200 Spectracube (Applied Spectral Imaging, Inc.) mounted on a Leica DMRBE microscope using a custom designed optical filter (SKY-1, Chroma Technology, Brattleboro, VT, U.S.A.). Using a Sagnac interferometer in the optical head, an interferogram was generated at all image points deduced from the optical path difference of the light which in turn depends on the wavelength ot the emitted fluorescence. By Fourrier transformation the spectrum was recovered. The spectral information was displayed by assigning red, green and blue colours to certain ranges of the spectrum. This red, green, blue (RGB) display renders chromosomes that were labelled with spectrally overlapping fluorochromes or fluorochrome combinations a similar colour. Based on the measurement of the spectrum for each pixel, however, a spectral classification algorithm was applied that allows the assignment of a pseudocolour to all points in the image that have the same spectrum. This algorithm forms the basis for chromosome identification by spectral karyotyping (Garini *et al.*, 1996; Schröck *et al.*, 1996). DAPI images were acquired from all metaphases analysed using a DAPI specific optical filter set.

RESULTS

Cytogenetic analysis

P1, female fetus

In routine chromosomal analysis after long term culture of amniotic fluid cells using GTG banding at a resolution level of 300–350 bands per genome, a normal female karyotype was found. A second chromosomal analysis of fetal blood showed the same result.

P2, male fetus

After long term culture of amniotic fluid cells chromosomal analysis using GTG banding at a resolution level of 350 bands per genome showed a numerically normal male karyotype with four derivative chromosomes (4, 11, 12 and 13). An interstitial deletion in the long arm of chromosome 4 was assumed, with the deleted material probably translocated into the long arm of chromosome 12. Further we found a terminal deletion in the short arm of chromosome 11 and additional material in the short arm of chromosome 13, assumed to be material from chromosome 11.

The second chromosomal analysis of fetal blood confirmed the finding of the CCR after GTG banding (Fig. 1).

The derivative chromosome 13 showed positive NOR staining in the proximal part of the short arm (Fig. 2).

Parents

Both parents had a normal karyotype.

Ultrasonographic findings

The male fetus revealed no characteristic malformation pattern.

The most striking abnormalities were the pathologic hectic movements of the male fetus. It was slightly growth retarded although still in the normal range (see Table 1), the profile was rather flat with micrognathia.

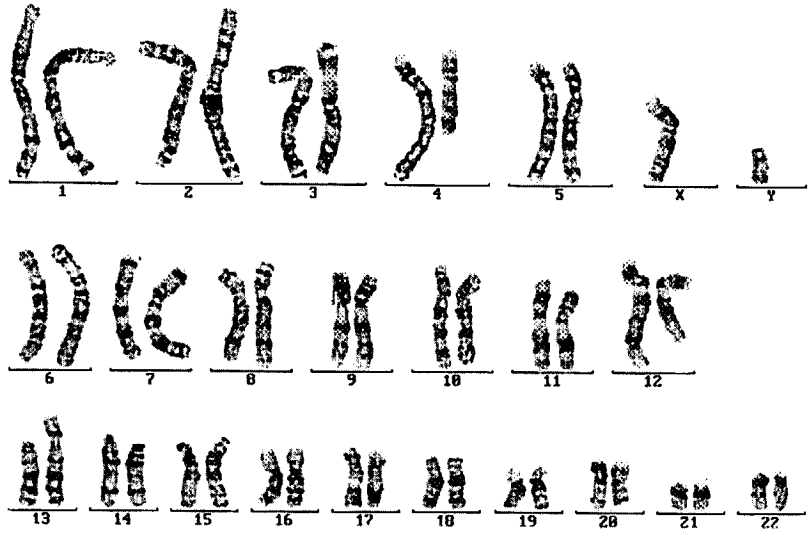

Fig. 1—GTG banded karyotype of the CCR

All these symptoms might have been regarded as still within the normal range but comparing male (Fig. 3A) and female fetus (Fig. 3B) they gained importance by the significant differences of the two children. Comparing all parameters of the two children by special diagnostic ultrasound investigation it was possible to ascertain the importance of even minor abnormalities of the male fetus.

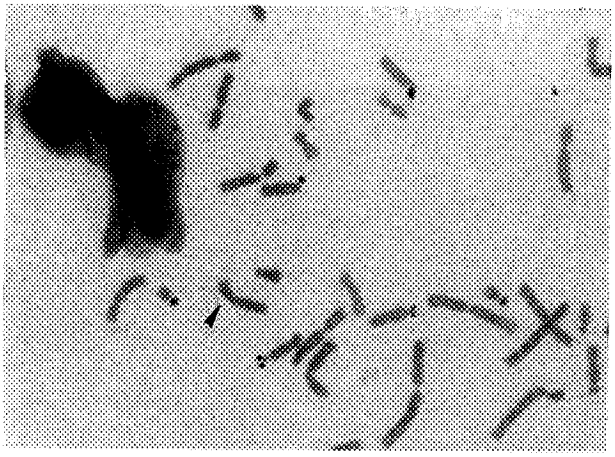

Fig. 2—Metaphase with NOR staining and a positive interstitial signal in the proximal part of the short arm (arrow) of the derivative chromosome 13

Table 1—Ultrasonographic findings at 22 weeks plus 5 days

Parameter	Male (cm)	Female (cm)
BPD	5.7	6.2
OFD	7.1	7.3
HC	21.0	22.3
ATD	5.4	6.1
ASD	5.4	6.0
FEM	3.5	4.0

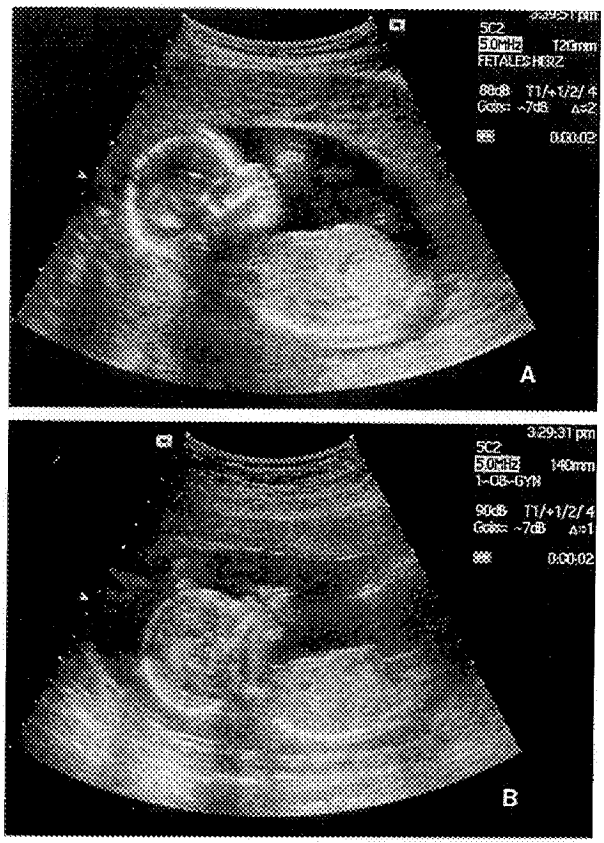

Fig. 3—A and B: Longitudinal ultrasound scans at 22 weeks plus 5 days of the male fetus (A) and the female fetus (B)

Molecular cytogenetic analysis

P2, male fetus

Using WCP4, the aberrant chromosome 4 showed hybridization signals along the entire euchromatic length, with the exception of a small part in the distal region of the long arm. One additional signal of chromosome 4 origin was detected in the distal short

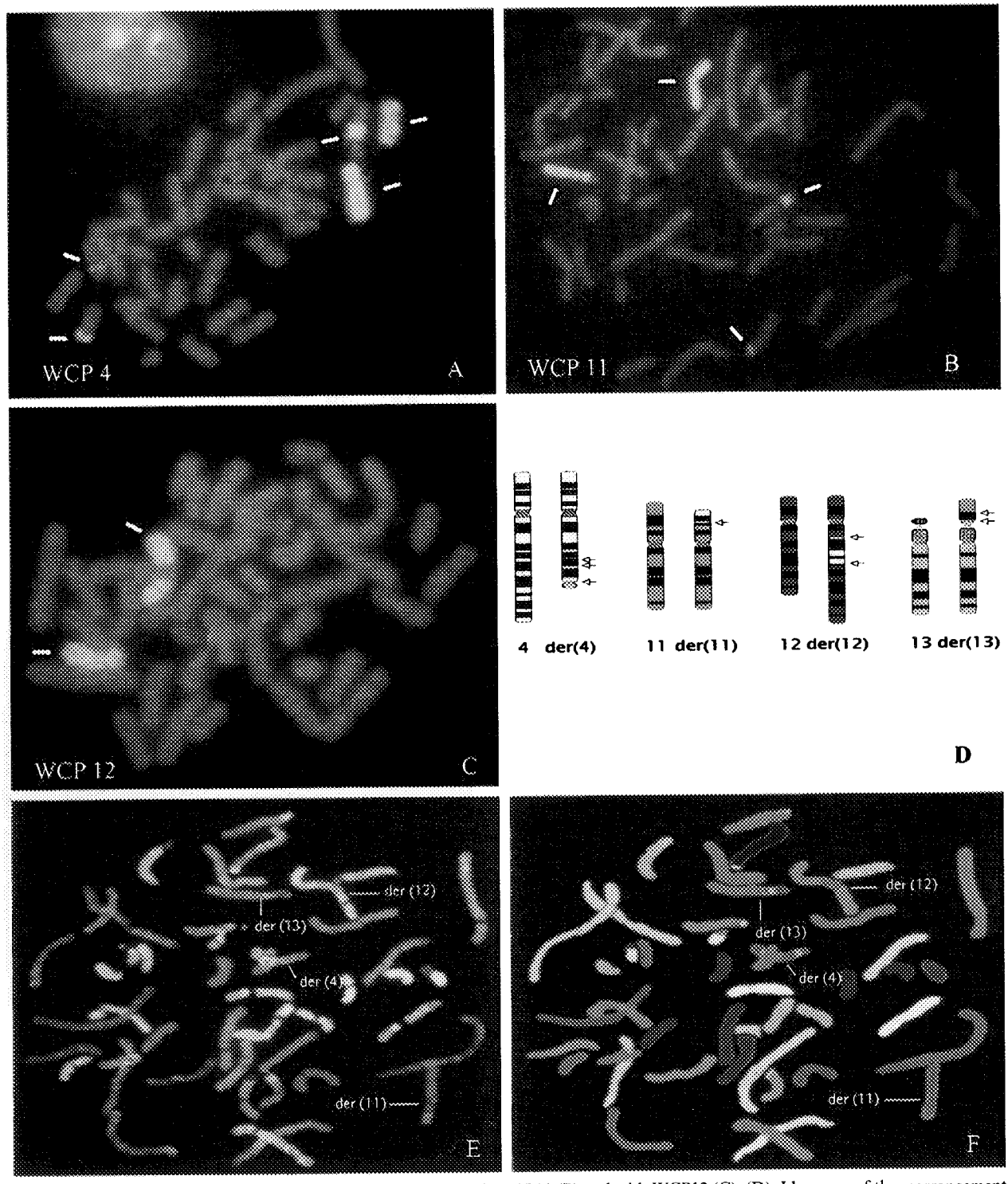

Fig. 4—A–C: FISH results in the male fetus with WCP4 (A), with WCP11 (B) and with WCP12 (C). (D): Ideogram of the rearrangement of the male fetus showing normal chromosomes 4, 11, 12 and 13 and their derivatives as interpreted after G banding and FISH analysis. The order of the segment from chromosome 13 on the der(4) was tentatively determined. Arrows show breakpoints. (E) and (F): SKY results: Metaphase spread after simultaneous hybridization of 24 combinatorially labelled chromosome painting probes showing the der(4), der(11), der(12), der(13), display colours (E), spectra based colours (F)

arm region of the aberrant chromosome 11. Further material from chromosome 4 was translocated and inserted interstitially into the long arm of chromosome 12. Additionally a small signal was present proximal in the short arm of chromosome 13 (Fig. 4A).

In addition to the normal chromosome 11 the whole chromosome 11 paint gave hybridization signals along the entire euchromatic length of the aberrant chromosome 11 except for the distal part of the short arm. One additional signal was detected on the

aberrant chromosome 13 in the distal region of the short arm and another one was present in the distal region of the deleted long arm of chromosome 4 (Fig. 4B).

After hybridization with WCP12, the aberrant chromosome 12 lacked signals in the middle of the long arm, whereas the rest of chromosome 12 was painted. No other signals were detected except on the normal chromosome 12 (Fig. 4C).

Analysing the rearrangement of the CCR after FISH we ascertained the absence of the telomeric region of the long arm of the derivative chromosome 4. One possible explanation is the translocation of the telomeric region 13p13 onto chromosome 4. This could not be verified because it is not possible to perform FISH for all human telomeres. So the order of the chromosome 13 segment terminal in the long arm of the derivative chromosome 4 could only be tentatively determined.

Comparison of the results of the FISH analysis and GTG banding ascertained the karyotype to be: 46,XY,t(4;11;12,13)(4pter-4q25::11p14-11p14::4q25-4q27::13p13-13pter; 11qter-11p14::4q33-4qter; 12pter-12q13::4q27-4q32::12q13-12qter; 13qter-13p12::4q33-4q32::11p14-11pter). ish der(4)(wcp4+,wcp11+); der(11)(wcp11+,wcp4+); der(12)(wcp12+,wcp4+); der(13)(wcp4+;wcp11+). It must be mentioned that the orientation of the small insertions (direct versus inverted) is not clear.

Figure 4D shows the ideogram of the normal and the rearranged chromosomes. Spectral karyotyping could detect the involvement of the chromosomes 4, 11, 12 and 13 in the CCR with eight breakpoints (Fig. 4E, F). In one hybridization, SKY could identify the translocation of chromosome 4 material to the distal part of the short arm of chromosome 11 and the insertion of chromosome 4 material into the long arm of chromosome 12. Also the translocation of material of chromosome 11 to the short arm of chromosome 13 was identified unambiguously. Additional material in the distal part of the long arm of the aberrant chromosome 4 was detected as well as additional material in the proximal short arm of chromosome 13, but for these two small regions the sofware was not able to assign the origin to a single chromosome. The additional material in chromosome 4q, however, was classified by SKY as either chromosome 11 or 15, which was identified previously by FISH to be of chromosome 11 origin. The additional material in the proximal region of 13 was classified as 15 or 4. Previous FISH identified it to be of chromosome 4 origin.

SKY detected no further involved chromosomes.

DISCUSSION

CCRs are normally first identified by standard chromosome banding techniques. However, complete characterization is not always possible using banding techniques alone due to their complexity. In these cases, FISH can provide additional information resulting in a more accurate definition of the rearrangement. The probes chosen for testing are usually determined by information from G banded analysis concerning the chromosomes involved.

In cases where FISH has been used for re-evaluation of G banding analyses, this has often resulted in an increased estimate of the number of breakpoints (Batista *et al.*, 1993; Verma *et al.*, 1993; Batista *et al.*, 1994; Fuster *et al.*, 1997). In our present case G banding showed that four chromosomes (4,11,12 and 13) were involved in the CCR with four rearrangements and six breakpoints. After combination of the traditional banding method and FISH we detected five rearrangements in the same four chromosomes. Eight breakpoints were proposed to explain these results. Most of the findings could only be detected with FISH, demonstrating the usefulness of this technique in characterizing chromosomal abnormalities that would be difficult to interpret correctly with classical cytogenetics alone.

In spite of the improvement of combination of traditional banding methods with FISH it is difficult to explain the CCR exactly in all details. One difficulty arises from the various possible origins of the telomeric region on the long arm from chromosome 4. Since the CCR seems to be stable we assume that a telomere is present. One possibility of this telomere is a translocation of a terminal segment 13p13 onto the derivative chromosome 4, which is below the level of resolution of G banding, FISH with whole chromosome paints and SKY. Another hypothesis is the loss of 13p13. For these data one explanation is the break and translocation of the terminal/telomeric region from chromosome 4 onto the derivative chromosome 11 and a *de novo* synthesis of a telomere on the derivative chromosome 4.

The presence of telomeres in chomosomes with presumptive terminal deletions has been demonstrated by FISH (Lin *et al.*, 1991; Park *et al.*, 1992). Wilkie *et al.* (1990) described a patient with α-thalassemia due to a terminal deletion on chromosome 16. DNA sequencing showed that $(TTAGGG)_n$ sequences were added directly at the site of the breakpoint. Later, Morin (1991) demonstrated that the enzyme telomerase recognizes a sequence at this breakpoint region on chromosome 16 which acts as a substrate for the addition of telomere sequences. A similar mechanism may well apply in our case. If the second hypothesis is correct, then strictly this CCR should be considered unbalanced. However, if the piece missing encompasses just the telomere and some closely neighbouring sequences, we could call this CCR functionally balanced. A panel of subtelomeric probes of the 13p terminal region would help to address this question, as well as further sequence data from the Human Genome Project.

As shown in the present case, SKY will be very useful for identifying chromosomal rearrangements that cannot be recognized by conventional banding. The identification of such a CCR by FISH using single chromosome paints is very time consuming. But SKY also has its limitations in the classification of involved

Table 2—Description of apparently balanced *de novo* CCRs detected at prenatal diagnosis

Chromosomes	No. of breakpoints	Indication	Fetal anomalies	Outcome	Reference
(4;11,12,13)	8	AMA, TP	+	Selective termination	Present case
(6;12;14,16)	9	Risk tri 21	+	Terminated	Phelan *et al.* (1998)
(5;6;11)inv(5)	4	Risk tri 21	−	Several minor anomalies	Ruiz *et al.* (1996)
(3;8)(6;7;18)	6	AMA	−	Hirschsprung disease, development delay	Ruiz *et al.* (1996)
(2;3;4;13)	5	Abnormal US	+	MCA	Mercier *et al.* (1996)
(3;4;10;17)	7	AMA	−	Terminated	Sikkema-Raddatz *et al.* (1995)
(2;5;18)	5	AMA	−	Normal at 3 years	Sikkema-Raddatz *et al.* (1995)
(1;3;4;9)	9	Low MSAFP	−	Terminated	Batista *et al.* (1993)
(7p;7q;14q)	3	AMA	−	Normal at 2 years	Kohler *et al.* (1986)
(4;6;15;16)	4	FH MR	+	Terminated	Kim *et al.* (1986)
(6;11;21)	4	FH NTD	−	Growth and speech development delay	Bogart *et al.* (1986)

CCR: complex chromosome rearrangement; AMA: advanced maternal age; TP: twin pregnancy; US: ultrasonogram; MSAFP: maternal serum alpha-fetoprotein; FH: family history; NTD: neural tube defect; MR: mental retardation; MCA: multiple congenital anomalies.

chromosomes. The resolution is about 1–2 Mb, as described by Macville *et al.* (1997) and sometimes small rearranged chromosomal segments cannot be classified reliably due to strong fluorescent signals of flanking chromosomal regions. This may be the difficulty in the classification of the translocated segments of chromosome 11 to chromosome 4 and of chromosome 4 to 13. In those cases, additional FISH has to be preformed with single chromosome painting probes or locus specific probes.

But in the future the sensitivity of SKY will be improved; new fluorochromes and other labelling schemes are being tested.

To our knowledge, the case of an apparently balanced *de novo* CCR reported here is the eleventh prenatally detected case. A summary of these cases is presented in Table 2. The rearrangements concern two to four chromosomes with three to nine breakpoints.

Five pregnancies were terminated (Kim *et al.*, 1986; Batista *et al.*, 1993; Sikkema-Raddatz *et al.*, 1995; Phelan *et al.*, 1998; present case); two of them showed no apparent anomalies at autopsy (Batista *et al.*, 1993; Sikkema-Raddatz *et al.*, 1995). Six pregnancies were pursued. Postnatal follow-ups showed two apparently normal patients at two and three years of age (Sikkema-Raddatz *et al.*, 1995; Kohler *et al.*, 1986). Bogart *et al.* (1986) described a child which was phenotypically normal at birth, but showed delayed growth as well as speech development at 2.5 years of age, thus demonstrating the need for long-term follow-up in assessing the consequences of apparently balanced CCRs. Of the last three cases cited above, one had multiple congenital anomalies including poorly differentiated ears, short neck, widely spaced nipples, hypospadias, bilateral hydrocele, supernumerary distal forearm crease, bilateral single palmar crease, overlapping fingers and metatarsus valgus. The infant was diagnosed during the neonatal period with Hirschsprung disease (Mercier *et al.*, 1996). One patient from Ruiz *et al.* (1996) also had Hirschsprung disease and was developmentally delayed and the other one had several minor anomalies such as a prominent forehead with some asymmetry, depressed nasal bridge, anteverted nares, undescended right testicle, mild fifth finger clinodactyly, prominent cutis marmorata, and diffuse hypotonia.

The review of the few prenatally diagnosed *de novo*, apparently balanced, CCRs showed the paucity of information and the difficulties in evaluating the risks for malformations, dysmorphic features and/or mental deficiencies.

More information and postnatal follow-up of prenatally detected *de novo* CCR cases are needed for more exact risk estimates and to determine the exact nature of the risk.

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